PATENT
Attorney Docket No.: 02307E-120110US
Client Ref. No.: UC 2001-510-2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 4699
ZUKER et al.	Examiner: Michael T Brannock
Application No.: 09/927,315	Technology Center/Art Unit: 1646
Filed: August 10, 2001	
For: MAMMALIAN SWEET TASTE RECEPTORS	DECLARATION UNDER 37 C.F.R. §1.132 OF CHARLES S. ZUKER
Customer No: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Charles S. Zuker, Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I received my Ph.D. in biology from Massachusetts Institute of Technology. I am currently a Professor and Investigator at Howard Hughes Medical Institute,

Departments of Biology and Neurosciences, School of Medicine, University of California at San Diego. I have been at this and related positions since 1986. A copy of my resume is attached as Exhibit A.

3. The invention of the above-referenced patent application provides for the first time a method for identifying compounds capable of activating or inhibiting sweet signal transduction in taste cells by detecting the functional effect of a candidate compound on a sweet taste receptor comprising a T1R3 polypeptide and a T1R2 polypeptide.

4. As a named invention of this patent application, I have read and am familiar with the contents of this application. In addition, I have read the Final Office Action, mailed June 3, 2004, received in the present case. It is my understanding that the Examiner believes that one of skill in the art would not know, even after reading this application, how to identify activators/inhibitors of sweet taste signaling when the sweet taste receptor is not present on the cell membrane. It is also my understanding that the Examiner further believes that a person skilled in the art would not know what G proteins may be used in connection with T1R3 and T1R2 for screening compounds capable of activating or inhibiting sweet taste signaling.

5. This declaration is provided to demonstrate that a person of ordinary skill in the art would know, particularly after reading the present application, how to practice the claimed method when the sweet taste receptor is not present on the cell membrane, and which G proteins may be used with a T1R3 polypeptide and a T1R2 polypeptide in the claimed screening method.

6. The Examiner cited the Lindemann reference (*Nature Medicine* **5**:381-382, 1999) to support his doubt that functional characteristics of a G-protein coupled taste receptor can be studied in a cell-free system. I believe, however, that the Examiner's reading of the Lindemann reference is overly narrow. The section of the reference the Examiner cited (*i.e.*, the middle paragraph of the first column on page 382) relates to several specific difficulties in assaying ligand specificity of a G-protein coupled receptor (GPCR) in a system where the GPRC is expressed in a heterologous host cell. While it is true that GPCR degradation, cell surface

expression, and correct protein conformation are all potential problems that could complicate the functional analysis in a cell-based assay system or even render such a cell-based assay system inoperable, this discussion does not stand for the position that a cell-free assay system cannot be used to properly assess the functional features of a G-protein coupled taste receptor.

7. There are various methods that can be used for detecting sweet taste signal transduction in a cell-free context, such as protein-protein binding assays, gel mobility shift assays, immunoassays, and enzymatic assays in a competitive or noncompetitive format. For example, the specification teaches measuring sweet taste signal transduction based on ligand-receptor binding, which is certainly suitable for practice in a cell-free assay system, such as the case where a sweet taste receptor is immobilized to a solid support.

8. This declaration is provided to further demonstrate that a person of ordinary skill in the art would know which G proteins, besides Gα15, may be used in connection with T1R3 and T1R2 for screening compounds capable of activating or inhibiting sweet taste signaling.

9. A number of G-proteins are known in the art as the promiscuous G-proteins that can couple a variety of GPCRs to downstream signaling effectors such as phospholipase C. For example, Offermanns and Simon (*J. Biol. Chem.*, **270**:15175-15180, 1995, attached as Exhibit B) describe promiscuous G-proteins Gα15 and Gα16, which can be activated by a wide variety of GPCRs. Gustducin is another G-protein that is known to be involved in both bitter and sweet signal transduction (*see, e.g.*, Wong *et al.*, *Nature* **381**:796-800, 1996, attached as Exhibit C) and therefore can be used in the present invention. For the purpose of studying the functional effects of a GPCR, chimeric G-proteins based on a promiscuous G-protein (*e.g.*, gustducin or Gα16) have also been made and used in cell-based assays. *See, e.g.*, Nelson *et al.*, *Cell*, **106**:381-390, 2001, reference C6 of IDS filed March 28, 2002.

10. Thus, a G-protein suitable for the present invention is not limited to Gα15. A person skilled in the art would know that other G-proteins, either naturally occurring or genetically modified, could be used for practicing the present invention.

11. Furthermore, it is often unnecessary to introduce an exogenous G-protcin into an assay system in order to practice the present invention. For instance, endogenous G-proteins exist in taste cells and naturally couple with the T1R3-T1R2 taste receptor in signal transduction. Another example is a cell-free assay system, where the T1R3-T1R2 receptor alone, without any G-protein, can be used to screen for a potential taste modulator.

12. In summary, it is my opinion that, upon after reading the present application, a person of ordinary skill in the art of molecular and cellular biology would know how to practice the claimed method to identify activators or inhibitors of sweet taste signal transduction when the sweet receptor is not present on the cell membrane, and which G proteins (besides G_15) may be suitable for use in the claimed method with a T1R3 polypeptide and a T1R2 polypeptide.

Date: NOVEMBER 2, 2004

By: _____
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Attachments (Exhibit A: Dr. Zuker's resume; Exhibit B: Offermanns and Simon, *J. Biol. Chem.*, **270**:15175-15180, 1995; Exhibit C: Wong *et al.*, *Nature* **381**:796-800, 1996)
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EDUCATION

INSTITUTION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Universidad Catolica de Valparaiso; Chile	B.Sc., Honors	1977	Biology
Massachusetts Inst. of Technology; Boston	Ph.D.	1983	Biology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE

1993 - present	Professor and Investigator; Howard Hughes Medical Institute Departments of Biology and Neurosciences, School of Medicine University of California, San Diego
1989 - 1992	Associate Professor and Associate Investigator Howard Hughes Medical Institute, UCSD
1986 - 1989	Assistant Professor; Department of Biology, UCSD
1983 - 1986	Postdoctoral Fellow; Department of Biochemistry; University of California, Berkeley
1977 - 1983	Graduate Student; Department of Biology; Massachusetts Institute of Technology

Honors and Keynote Lectures (selected)

Whitaker Health Sciences Fund Fellow, Massachusetts Inst. of Technology, 1979-1980
Whitaker Health Sciences Fund Fellow, Massachusetts Inst. of Technology, 1981-1982
European Molecular Biology Organization Fellow, 1983
Jane Coffin Childs Memorial Fund for Medical Research Fellow, 1984-1986
McKnight Foundation Fund for Neuroscience Award, 1988-1991
Monsanto Speaker, St. Louis University, St Louis, MO, 1991
Broadhurst Foundation visiting lecturer, Cambridge, MA, 1991
Institute Speaker, Scripps Research Institute, La Jolla, CA, 1992
Keynote speaker, Stanford Neurosciences Program Retreat, Monterey, CA,1992
Pew Scholars Award, 1988-1992
Alfred P. Sloan Award in Neurosciences, 1988-1990
March of Dimes Basil O'Connor Award, 1989-1991
Merck Lecturer, UC Berkeley 1992
Institute speaker, Roche Institute of Molecular Biology, Nutley, NJ, 1993
Keynote Speaker, Pharmacological Sciences Program, Vanderbilt University, Nashville, TN, 1994
Keynote Speaker, Stanford Medical Scientist Training Program, Stanford University CA, 1994
Lecturer in the Life Sciences, Northwestern University Medical School, Chicago, IL 1994

Howard Hughes Medical Institute, Lecture series to Institute employees, Howard Hughes Medical Institute, Chevy Chase, MD, 1996
Keynote Speaker, FASEB Summer Conference on "The Biology and Chemistry of Vision", Keystone, CO, 1997
Keynote Speaker, U. Penn Graduate programs in Biochemistry, Molecular Biology and Pharmacology. Philadelphia, 1998
Cogan Award, Association for Research in Vision and Ophthalmology, 1998
University Lecturer, UT Southwestern Medical School, 1999
Alcon Award for outstanding contributions to vision research, 1999
American Academy of Arts and Sciences, 2000

Study Sections and Advisory Boards (selected):
Member, Scientific Advisory Board, Pew Latin American Scholars Program, 1990 - present
Mechanisms of Development, 1991-present
Neuron, 1995-present
Member, American Cancer Society Postdoctoral Research Selection Committee, 1995-1999
Member, Scientific Advisory Board, Schepens Research Institute, Harvard University, Cambridge, MA, 1995 - present
Member, Review Panel, Howard Hughes Medical Institute International Grants Program, 1996
Member, National Research Council/ National Academy of Sciences advisory committee for the US and HHMI program in Latin America, 1997-
National Advisory Committee of The Pew Scholars Program in the Biomedical Sciences, 1997-
Member, NIH Visual Sciences C study section, Bethesda, MD, 1997-2000
Member, NIDCD Strategic Planning committee 1999-
Damon Runyon-Walter Winchell Cancer Fund Scientific Advisory Committee, 1999-
Current Biology, 2000-
Steering Committee, Alliance for Cellular Signaling, 2000-
Advisory board, Pew program in Science and Society, 2001-
Advisory board, NIH-wide initiative on mouse mutagenesis, 2001-

Publications (selected):

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$G\alpha_{15}$ and $G\alpha_{16}$ Couple a Wide Variety of Receptors to Phospholipase C*

(Received for publication, March 27, 1995)

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The murine G-protein α-subunit $G\alpha_{15}$ and its human counterpart $G\alpha_{16}$ are expressed in a subset of hematopoietic cells, and they have been shown to regulate β-isoforms of inositide-specific phospholipase C. We studied the ability of a variety of receptors to interact with $G\alpha_{15}$ and $G\alpha_{16}$ by cotransfecting receptors and G-protein α-subunits in COS-7 cells. Activation of β_2 adrenergic and muscarinic M_2 receptors in cells expressing the receptors alone or together with $G\alpha_q$, $G\alpha_{11}$, or $G\alpha_{14}$ led to a very small stimulation of endogenous phospholipase C. However, when the receptors were coexpressed with $G\alpha_{15}$ and $G\alpha_{16}$, addition of appropriate ligands caused a severalfold increase in inositol phosphate production which was time- and dose-dependent. A similar activation of phospholipase C was observed when several other receptors which were previously shown to couple to members of the G_i and G_s family were coexpressed with $G\alpha_{15/16}$. In addition, stimulation of inositol phosphate formation via receptors naturally coupled to phospholipase C was enhanced by cotransfection of $G\alpha_{15}$ and $G\alpha_{16}$. These data demonstrate that $G\alpha_{15}$ and $G\alpha_{16}$ are unique in that they can be activated by a wide variety of G-protein-coupled receptors. The ability of $G\alpha_{15}$ and $G\alpha_{16}$ to bypass the selectivity of receptor G-protein interaction can be a useful tool to understand the mechanism of receptor-induced G-protein activation. In addition, the promiscuous behavior of $G\alpha_{15}$ and $G\alpha_{16}$ toward receptors may be helpful in finding ligands corresponding to orphan receptors whose signaling properties are unknown.

A wide variety of hormones and neurotransmitters regulate cellular functions by binding to transmembranous receptors which couple to and activate heterotrimeric guanine nucleotide binding proteins (G-proteins).[1] Receptor-activated G-proteins then regulate different cellular effectors, such as specific enzymes and ion channels (1–4). Sixteen mammalian genes encoding G-protein α-subunits, which define the individual G-proteins, have been identified, and they have been grouped into four families, $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$, according to sequence homologies (5). Many of the factors which determine the specificity of G-protein-mediated signal transduction are still unknown. Nonetheless, the selective coupling of an activated receptor to a distinct pattern of G-proteins is regarded as an important requirement to achieve accurate signal transduction. For example, receptors which upon activation lead to stimulation of adenylyl cyclase primarily couple to G_s, whereas the receptor-mediated pertussis toxin-insensitive activation of phospholipase C is due to the coupling of receptors to members of the G_q family (6–8).

The G_q family consists of five members whose α-subunits show different expression patterns. Whereas $G\alpha_q$ and $G\alpha_{11}$, which are 88% identical, seem to be almost ubiquitously expressed and are primarily responsible for coupling receptors in a pertussis toxin-insensitive manner to phospholipase C β-isoforms (7–9), the expression of $G\alpha_{14}$, which is 81% identical with $G\alpha_q$, is more restricted (10). The human $G\alpha_{16}$ and its murine counterpart $G\alpha_{15}$ are only expressed in a subset of hematopoietic cells (10, 11). $G\alpha_{15}$ and $G\alpha_{16}$, which are 85% identical, have been placed into the G_q family since their sequences show the highest similarity toward $G\alpha_q$ (57%). All five members of the $G\alpha_q$ family share functional properties, *i.e.* they can regulate the β-isoforms of phospholipase C (12–14). Purified $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{16}$ indistinguishably activate different isoforms of phospholipase C-β in a reconstituted system (15, 16). Recent data, however, demonstrate that receptors for interleukin 8 and C5a interact selectively with G_{16} but not with G_q and G_{11} (17–19), demonstrating that there are differences among members of the G_q family with regard to receptor interaction. In the present study, we show that a wide variety of structurally and functionally different receptors couple to G_{15} and G_{16} but not to other members of the G_q family, indicating that G_{15} and G_{16} are unique, *i.e.* they possess the ability to nonselectively couple a large variety of receptors to phospholipase C.

EXPERIMENTAL PROCEDURES

Materials—Carbachol, isoproterenol, dopamine, 8-hydroxy-2-(di-*n*-propylamino)tetralin, thrombin, serotonin, [Arg^8]vasopressin, [D-Ala^2, *N*-MePhe^4,Gly^5-ol]enkephalin, 11α,9α-epoxymethano-PGH_2 (U46619), and *N*-formyl-Met-Leu-Phe were from Sigma. CGS-21680 was from RBI (Natick, MA) and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) was from Life Technologies, Inc.

Transient Transfection and Labeling of COS-7 Cells—COS-7 cells were cultured as described (13). For transfection experiments, cells were seeded in 24-well plates at a density of 4×10^4 cells per well and grown overnight. Cells were then washed with phosphate-buffered saline, and 0.4 μg of DNA mixed with 2 μl of lipofectamine (Life Technologies, Inc.) in 0.25 ml of Opti-MEM was added to each well. In cotransfection experiments with two different plasmids, 0.2 μg of each plasmid was added. In control experiments, the total amount of DNA was maintained constant by adding DNA from a vector encoding β-galactosidase. After 5 h at 37 °C, 0.25 ml of DMEM containing 20% (v/v) fetal bovine serum was added to each well. About 24 h after transfection, cells were labeled for 20–24 h with 120 pmol of *myo*-[2-^{3}H]inositol (758.5 GBq/mmol; Du Pont NEN) per well as described (13).

Determination of Inositol Phosphate Levels—Labeled cells were washed with phosphate-buffered saline and then incubated for 10 min at 37 °C with 0.25 ml of inositol-free DMEM containing 10 mM LiCl.

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‡ Recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

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[1] The abbreviations used are: G-protein, heterotrimeric guanine nucleotide-binding protein; DMEM, Dulbecco's modified Eagle's medium; fMLP, *N*-formyl-methionine-leucine-phenylalanine; C5a, active cleavage product of the complement factor 5; 5-HT, 5-hydroxytryptamine (serotonin); U46619, 11α,9α-epoxymethano-PGH_2.

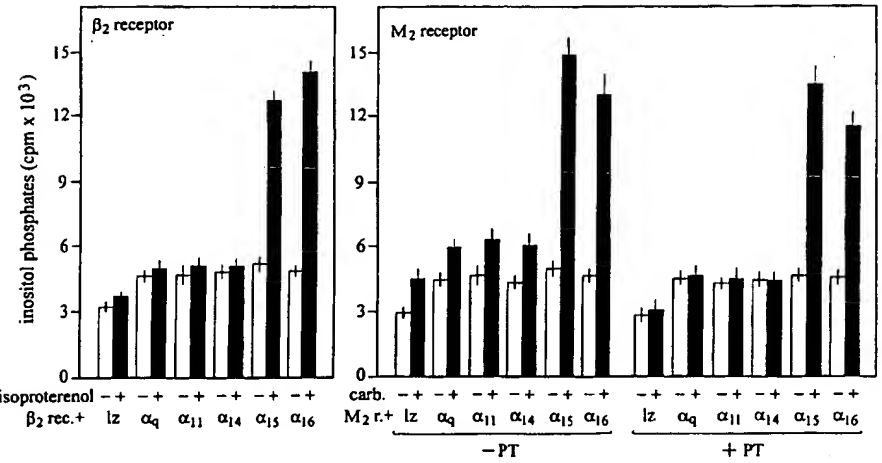

FIG. 1. **Accumulation of inositol phosphates in COS-7 cells that coexpress the β_2 adrenergic or the M_2 muscarinic receptor and Gα subunits.** COS-7 cells were cotransfected with cDNAs encoding the the β_2 adrenergic (*left panel*) or the M_2 muscarinic receptor (*right panel*) and cDNAs encoding β-galactosidase (*lz*) or α-subunits of the $G\alpha_q$ family, $G\alpha_q$ (α_q), $G\alpha_{11}$ (α_{11}), $G\alpha_{14}$ (α_{14}), $G\alpha_{15}$ (α_{15}), and $G\alpha_{16}$ (α_{16}) as described under "Experimental Procedures." After 48 h, [^{3}H]inositol-labeled cells were incubated in the absence (−; *open bars*) or presence (+) of 10 μM isoproterenol (*closed bars, left panel*) and 10 μM carbachol (*carb.*; *closed bars, right panel*) for 20 min, and levels of inositol phosphates were determined as described. Cells which were cotransfected with the M_2 muscarinic receptor cDNA and Gα subunit cDNAs were processed as described (−*PT*) or were pretreated with 100 ng/ml pertussis toxin (+*PT*) for 18 h prior to incubation with ligand. Shown are mean values of triplicates ± S.D.

Thereafter, medium was aspirated, and the indicated agents were added in DMEM containing 10 mM LiCl. Inositol phosphate formation was stopped after 20 min by removing the medium and adding 0.2 ml of 10 mM ice-cold formic acid. After keeping the samples on ice for 20 min, 0.45 ml of 10 mM NH_4OH was added, and the whole sample was loaded onto a column containing 0.75 ml of anion exchange resin (AG 1-X8; Bio-Rad) equilibrated with 5 mM borax and 60 mM sodium formate. Total inositol phosphates were then separated and measured as described (20). If not stated otherwise, measurements were done in triplicate representing three independently transfected wells.

Determination of Cellular cAMP Levels—For determination of cAMP levels, cells were grown and transfected in 24-well plates as described. 48 h after transfection, cells were preincubated for 15 min with DMEM containing 300 μM 3-isobutyl-1-methylxanthine and 20 μM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20–1724). Thereafter, medium was replaced by DMEM containing both phosphodiesterase inhibitors and the indicated concentrations of ligands. At the end of this treatment (20- min incubation time), the reaction was stopped by aspiration of the medium and addition of 150 μl of ice-cold 10% (w/v) trichloroacetic acid. Samples were kept for 10 min on ice, and 90 μl of 1 M Tris (pH 9.8) was added to neutralize the sample. cAMP was determined by the competitive binding assay (21, 22). Briefly, 100 μl of the sample were incubated for 2 h with 2 pmol of [8-^{3}H]cAMP (925 Gbq/mmol; Amersham) and 62.5 μg of cAMP-dependent protein kinase purified from porcine heart (Sigma) in a final volume of 200 μl at 4 °C. Thereafter, 100 μl of 4% (w/v) charcoal in 5 mM EDTA and 50 mM Tris-HCl (pH 7.5) was added, and samples were immediately centrifuged for 2 min at 12,000 × *g*. To determine the amount of [8-^{3}H]cAMP bound to the binding protein, supernatants were counted in a liquid scintillation counter. Each experiment was calibrated by running a set of cAMP standards along with the unknown test sample. For the standard samples, the log of total counts/min bound was plotted *versus* the log of total cAMP per sample (labeled plus unlabeled), and the amount of cAMP in the test sample was calculated from the resulting standard curve (21). Assays were done in triplicate representing three independently transfected wells.

COS Cell Expression Vectors—cDNAs corresponding to G-protein α-subunits $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, and $G\alpha_{16}$ were carried by the cytomegalovirus vector pCIS (12, 13, 17, 19, 20). A β-galactosidase construct inserted into pCIS was used as a transfection control. cDNAs encoding the human muscarinic M_2, the human β_2 adrenergic, the murine 5-$HT_{1C/2C}$, and the human thrombin receptors were in the vector pCIS. The human dopamine D_{1A} construct was in pCMV5 (23), the rat μ opioid receptor and the human adenosine A_{2A} receptor encoding cDNAs were in pRc/CMV (Invitrogen), the human vasopressin V_2 receptor and the human fMLP receptor cDNAs were in the vector pcDNAI/Amp (Invitrogen), the human 5-HT_{1A} receptor construct was in pSVL (Pharmacia Biotech Inc.), the cDNA encoding the human vasopressin V_{1A} receptor was carried by the pcD3 vector (24), and the human thromboxane A_2 receptor cDNA was in pCDM8 (Invitrogen).

RESULTS

In order to study the interaction of different receptors and G-proteins of the G_q family, cDNA clones encoding receptors and G-protein α-subunits were transiently cotransfected into COS-7 cells, and inositol phosphate production in response to receptor ligands was measured. First, we tested the β_2 adrenergic and the M_2 muscarinic receptor, which have been shown to couple primarily to G_s and $G_{i/o}$, respectively (25–28), to determine if they can mediate ligand-dependent inositol phosphate production in COS-7 cells (Fig. 1). When both receptors were expressed alone, there was a slight increase in the inositol phosphate production in response to the respective ligands. In the case of the muscarinic M_2 receptor, this increase could be blocked by pretreatment of cells with pertussis toxin and is presumably mediated by βγ-subunits of G_i (29). Cotransfection of G-protein α-subunits $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{14}$, all of which have been shown to be expressed at high levels and to mediate receptor-dependent phospholipase C activation in COS cells (12, 13, 17, 19, 20), slightly increased the basal inositol phosphate production, but had no effect on the ligand-dependent increase in inositol phosphate formation. In contrast, ligand-dependent inositol phosphate production was severalfold enhanced when both receptors were cotransfected with $G\alpha_{15}$ and $G\alpha_{16}$ (Fig. 1).

Fig. 2 shows that the β_2 adrenergic or the M_2 muscarinic receptor-mediated increase in inositol phosphate production in cells cotransfected with $G\alpha_{15}$ or $G\alpha_{16}$ was linear with time for at least 30 min. Isoproterenol- and carbachol-induced inositol phosphate formation in cells transiently expressing $G\alpha_{15}$ or $G\alpha_{16}$ and the corresponding receptors was concentration-dependent (Fig. 3). Half-maximal and maximal effects of carbachol were observed at concentrations of 0.1 and 1–10 μM, whereas isoproterenol-stimulated inositol phosphate production was half-maximal and maximal at concentrations of 0.3 and 10 μM, respectively. We then tested the ability of isoproterenol and carbachol to stimulate inositol phosphate formation in the presence of $G\alpha_{15}$ and $G\alpha_{16}$ in order to test if the ligand concentration dependence was in the same range as the dosage required to regulate the natural effector target of their

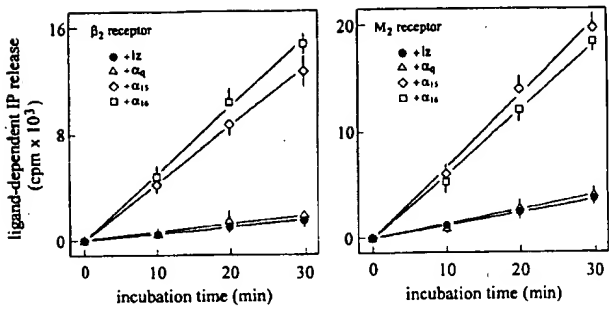

FIG. 2. **Time course of the β_2 adrenergic and M_2 muscarinic receptor-mediated inositol phosphate formation in COS-7 cells.** COS-7 cells were cotransfected with cDNAs encoding the β_2 adrenergic (*left panel*) or the M_2 muscarinic receptor (*right panel*) and cDNAs encoding β-galactosidase (●, lz) or $G\alpha_q$ (Δ, α_q), $G\alpha_{15}$ (◇, α_{15}), and $G\alpha_{16}$ (□, α_{16}) as described under "Experimental Procedures." Cells were incubated in the absence or presence of 10 μM isoproterenol (*left panel*) and 10 μM carbachol (*right panel*) for the indicated time periods (*abscissa*), and released inositol phosphates were measured as described. Shown is the ligand-dependent inositol phosphate release, and data points represent mean values of triplicates ± S.D.

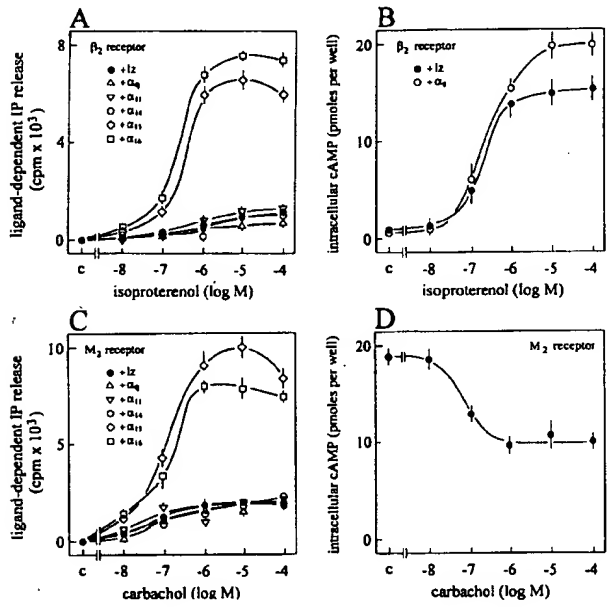

FIG. 3. **Accumulation of inositol phosphates and cAMP in COS-7 cells transfected with the β_2 adrenergic or M_2 muscarinic receptor cDNAs.** *A* and *C*, COS-7 cells were cotransfected with cDNAs encoding the β_2 adrenergic (*A*) or the M_2 muscarinic receptor (*C*) and cDNAs encoding β-galactosidase (●, lz) or α-subunits of the $G\alpha_q$ family, $G\alpha_q$ (Δ, α_q), $G\alpha_{11}$ (▽, α_{11}), $G\alpha_{14}$ (○, α_{14}), $G\alpha_{15}$ (◇, α_{15}), and $G\alpha_{16}$ (□, α_{16}) as described under "Experimental Procedures." Cells were then incubated with the indicated concentrations of isoproterenol (*A*) and carbachol (*C*), and the ligand-dependent inositol phosphate formation was determined as described. *B*, COS-7 cells were cotransfected with cDNAs encoding the β_2 adrenergic receptor and β-galactosidase (●, lz) or $G\alpha_s$ (○, α_s). Cells were incubated at increasing concentrations of isoproterenol for 20 min, and cellular cAMP content was determined as described under "Experimental Procedures." *D*, COS-7 cells were cotransfected with cDNAs encoding the the β_2 adrenergic and M_2 muscarinic receptor (0.2 μg of each per well). Transfected cells were incubated with 2 μM isoproterenol and the indicated concentrations of carbachol (*abscissa*) for 20 min, and the carbachol-induced decrease in cellular cAMP content was measured as described. Basal cAMP levels (in the absence of any ligand) were 1.2 ± 0.2 pmol per well. Values are mean values ± S.D.

respective receptors. Therefore, in a set of parallel experiments we measured the effect of increasing concentrations of both ligands on the cellular cAMP content in COS-7 cells transfected with the β_2 or the M_2 receptor cDNAs. Isoproterenol induced an increase in the intracellular cAMP content in cells expressing the β_2 receptor, and the extent of this effect was slightly increased in cells coexpressing $G\alpha_s$. In order to study the M_2 receptor-mediated decrease in intracellular cAMP, cells were cotransfected with the muscarinic M_2 receptor and the β_2 adrenergic receptor. When cAMP levels were increased through the stimulation of the β_2 receptor, a carbachol-dependent decrease of the cAMP content could be observed. In both cases, effects mediated by the β_2 and the M_2 receptor were dose-dependent and occurred with a very similar dose-response relationship as the $G\alpha_{15}/G\alpha_{16}$-dependent stimulation of phospholipase C via both receptors (Fig. 3). This shows that the activated β_2 and the M_2 receptor regulated the intracellular cAMP content in COS-7 cells with a very similar efficacy as they induced inositol phosphate formation when cotransfected with $G\alpha_{15}$ and $G\alpha_{16}$ into COS-7 cells.

To further determine the spectrum of receptors able to activate G_{15} and G_{16}, we cotransfected COS-7 cells with cDNAs of a variety of different receptors alone or together with $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, or $G\alpha_{16}$. We then measured the effect of increasing agonist concentrations on inositol phosphate formation. Fig. 4 shows the results obtained with three receptors which are naturally coupled to the stimulation of adenylyl cyclase, the vasopressin V_2, the dopamine D_{1A}, and the adenosine A_{2A} receptor (23, 30–32). When these receptors were expressed alone, a small dose-dependent increase in the inositol phosphate formation could be observed. In cells coexpressing the receptors and $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{14}$, the ligand-dependent inositol phosphate production was the same as in cells transfected with the receptors alone. However, when the vasopressin V_2, the dopamine D_{1A}, and the adenosine A_{2A} receptors were coexpressed with $G\alpha_{15}$ or $G\alpha_{16}$, the ligand-dependent inositol phosphate formation increased severalfold.

We then tested the ability of $G\alpha_{15}$ and $G\alpha_{16}$ to couple receptors, which activate G_i and G_o proteins, to the production of inositol phosphates (Fig. 5). The μ opioid receptor, the 5-HT_{1A} receptor, and the fMLP receptor, which have been shown to activate G-proteins of the G_i family (33–35), also mediated a small increase in the formation of inositol phosphates when expressed alone. No significant increase in the ligand-dependent inositol phosphate formation was observed when the receptors were coexpressed with $G\alpha_q$ or $G\alpha_{11}$. Coexpression of the fMLP or the 5-HT_{1A} receptor but not the μ opioid receptor and $G\alpha_{14}$ slightly increased the ligand-dependent inositol phosphate production. Ligand-induced release of inositol phosphates was again markedly increased when the receptors were coexpressed with $G\alpha_{15}$ and $G\alpha_{16}$, indicating that the receptors can couple to $G\alpha_{15}$ and $G\alpha_{16}$, but not to $G\alpha_q$ or $G\alpha_{11}$.

To prove that receptors which activate phospholipase C in a pertussis toxin-insensitive manner by coupling to $G\alpha_{q/11}$ can also activate $G\alpha_{15}$ and $G\alpha_{16}$, we expressed the thrombin, the thromboxane A_2, the vasopressin V_{1A}, and the 5-$HT_{1C/2C}$ receptors (24, 36–38) together with $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, and $G\alpha_{16}$ in COS-7 cells (Fig. 6). Since COS cells express $G\alpha_q$ and $G\alpha_{11}$ (13), the effects of cotransfected $G\alpha_q$ family members on the inositol phosphate formation mediated by phospholipase C-linked receptors is much weaker, as the receptors can interact with endogenous $G\alpha_q$ and $G\alpha_{11}$. Nevertheless, the tested phospholipase C-coupled receptors mediated the stimulation of inositol phosphate production, and the ligand-dependent portion could be significantly increased when the receptors were coexpressed with $G\alpha_q$ family members, including $G\alpha_{15}$ and $G\alpha_{16}$.

Thus, different receptors which couple to G_s, G_i, and G_q family members can be functionally linked to endogenous phospholipase C when coexpressed with $G\alpha_{15}$ and $G\alpha_{16}$ in COS cells, indicating that they all activate $G\alpha_{15}$ and $G\alpha_{16}$.

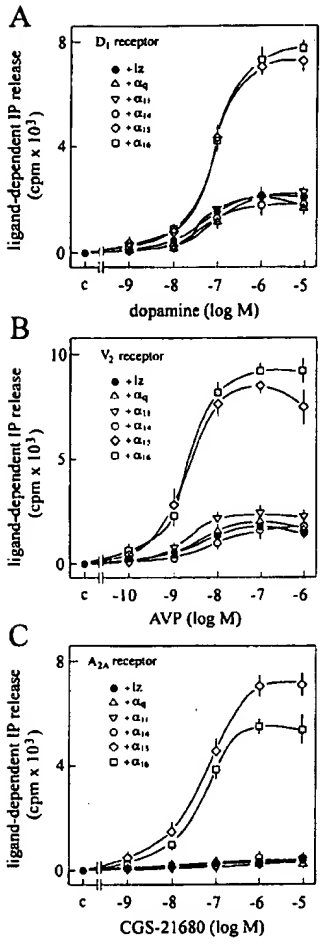

FIG. 4. **Formation of inositol phosphates in COS-7 cells coexpressing vasopressin V_2, dopamine D_1, or adenosine A_{2A} receptors and G-protein α-subunits.** COS-7 cells were cotransfected with cDNAs encoding the dopamine D_1 receptor (*A*), the vasopressin V_2 (*B*), or the adenosine A_{2A} receptor (*C*) and cDNAs encoding β-galactosidase (●, lz) or α-subunits of the $G\alpha_q$ family, $G\alpha_q$ (Δ, α_q), $G\alpha_{11}$ (▽, α_{11}), $G\alpha_{14}$ (○, α_{14}), $G\alpha_{15}$ (◇, α_{15}) and $G\alpha_{16}$ (□, α_{16}) as described under "Experimental Procedures." Cells were then incubated with the indicated concentrations of dopamine (*A*), [Arg^8]vasopressin (*AVP*; *B*), and CGS-21680 (*C*), and the ligand-dependent inositol phosphate formation was determined as described. Shown are mean values of triplicates ± S.D.

DISCUSSION

In this paper, we demonstrate that $G\alpha_{15}$ and $G\alpha_{16}$ are capable of coupling a variety of receptors to the stimulation of inositol phosphate formation when coexpressed with the receptor in COS-7 cells. Receptors which are not linked to a pertussis toxin-insensitive regulation of phospholipase C under physiological conditions mediated little increase in inositol phosphate formation when expressed alone. This increase was not altered by coexpression of $G\alpha_q$ and $G\alpha_{11}$. In some cases, coexpression of $G\alpha_{14}$ led to a slight increase of the basal ligand-dependent inositol phosphate formation (Fig. 5). All receptors tested gained the ability to mediate a severalfold increase in the inositol phosphate production when they were coexpressed with $G\alpha_{15}$ and $G\alpha_{16}$, indicating that they functionally interacted with $G\alpha_{15}$ and $G\alpha_{16}$. In addition, the inositol phosphate formation mediated by several receptors which are physiologically linked to phospholipase C in a pertussis toxin-insensitive manner, could be increased by coexpression of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$, and $G\alpha_{16}$. Thus, these receptors were able to act through all the transfected $G\alpha$ subunits.

The receptors shown to activate $G\alpha_{15}$ and $G\alpha_{16}$ represent a wide spectrum of structurally and functionally different ligand binding proteins, demonstrating that $G\alpha_{15}$ and $G\alpha_{16}$ can be activated by a wide variety of receptors which serve very different functions under physiological conditions. This appears to be a unique feature of $G\alpha_{15/16}$; other G-proteins usually are selectively activated by a defined spectrum of receptors. A certain degree of specificity in the receptor G-protein interaction is regarded as a prerequisite for proper signal transduction. Thus, a G-protein which can nonselectively link functionally different receptors in a given cell to the same effector would be thought to produce inappropriate signaling and block specific intracellular information processing pathways. However, $G\alpha_{15}$ and $G\alpha_{16}$ exhibit very restricted expression patterns. Expression has only been shown in a subset of hematopoietic cells, and especially at premature stages in different cell lineages (11, 39). Therefore, coupling of most of the tested receptors to $G\alpha_{15}$ and $G\alpha_{16}$ in the COS cell system is presumably without direct physiological significance, since many of these receptors and $G\alpha_{15}$ and $G\alpha_{16}$ may not be coexpressed *in vivo*.

The chemokine receptors for C5a and interleukin 8 have been shown to activate $G\alpha_{16}$, but not $G\alpha_q$ and $G\alpha_{11}$ (17–19), and they are known to be expressed in mature cells of the immune system, especially in leukocytes. Therefore, they have been implicated in the physiological regulation of $G\alpha_{15/16}$ activity. Whereas chemokine receptors for interleukin 8, C5a, and fMLP can undoubtedly couple to $G\alpha_{15}$ and $G\alpha_{16}$ (17–19, Fig. 5), our current work indicates that this ability is not restricted to this receptor class, but rather due to the unique properties of $G\alpha_{15/16}$. In addition, expression of chemokine receptors and $G\alpha_{15/16}$ seems to be regulated in a reciprocal manner during leukocyte development. For example, in undifferentiated HL-60 cells which express high levels of $G\alpha_{16}$, chemokine receptors are absent or at low levels, whereas chemokine receptor expression increases and the expression of $G\alpha_{16}$ dramatically decreases during differentiation of HL-60 cells (11, 40–42). The effects of chemokine receptors in myeloid cells are mainly pertussis toxin-sensitive (43–45), indicating that they are mediated by G_i-type G-proteins. Recently it has been shown that the fMLP receptor primarily functions through pertussis toxin-sensitive G-proteins even when stably expressed in undifferentiated HL-60 cells where $G\alpha_{16}$ is present (46). Chemokine receptors probably interact with $G\alpha_{15}$ and $G\alpha_{16}$ under some conditions *in vivo*; it is, however, tempting to speculate that the main receptors physiologically coupled to $G\alpha_{15}$ and $G\alpha_{16}$ are still to be identified. They might be involved in the regulation of growth and differentiation of hematopoietic cells, and $G\alpha_{15/16}$ may allow the coupling of diverse receptors to the stimulation of phospholipase C.

Since a variety of receptors can activate $G\alpha_{15}$ and $G\alpha_{16}$ while being unable to activate $G\alpha_q$ and $G\alpha_{11}$, the question arises of which structural determinants of $G\alpha_{15}$ and $G\alpha_{16}$ are responsible for their ability to become activated by many different receptors. The carboxyl-terminal 55 amino acids of $G\alpha_{15}$ and $G\alpha_{16}$ are most divergent from $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{14}$ (9–11). This region includes the very carboxyl terminus of the α-subunit which has been shown to affect receptor specificity (47, 48). Interestingly, $G\alpha_{15}$ and $G\alpha_{16}$ possess a unique insert of several amino acids including different charged residues between helix α4 and helix α5 which are just adjacent to a region homologous to residues 311–329 of transducin (10, 11, 49). This region of transducin has also been implicated in the interaction with receptor (50). However, the situation appears to be more complicated as shown by a recent study in which different chimeras between $G\alpha_{11}$ and $G\alpha_{16}$ were examined for their ability to

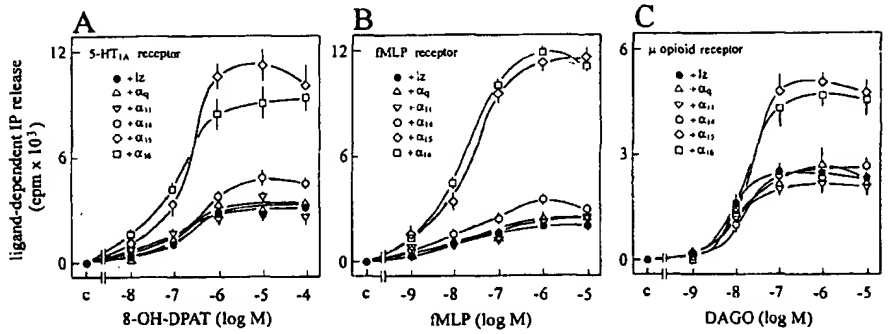

FIG. 5. **Accumulation of inositol phosphates in COS-7 cells cotransfected with cDNAs encoding the 5-HT_{1A}, the fMLP or the μ opioid receptor, and G-protein α-subunits.** COS-7 cells were cotransfected with cDNAs encoding the 5-HT_{1A} (*A*), the fMLP (*B*), or the μ opioid receptor (*C*) and cDNAs encoding β-galactosidase (●, lz) or α-subunits of the $G\alpha_q$ family, $G\alpha_q$ (Δ, α_q), $G\alpha_{11}$ (▽, α_{11}), $G\alpha_{14}$ (○, α_{14}), $G\alpha_{15}$ (◇, α_{15}), and $G\alpha_{16}$ (□, α_{16}) as described under "Experimental Procedures." Cells were then incubated with increasing concentrations of 8-hydroxy-2-(di-*n*-propylamino)tetralin (*A*), fMLP (*B*), and [D-Ala²,*N*-MePhe⁴,Gly⁵-ol]enkephalin (*C*), and the ligand-dependent inositol phosphate formation was determined as described. Shown are mean values of triplicates ± S.D.

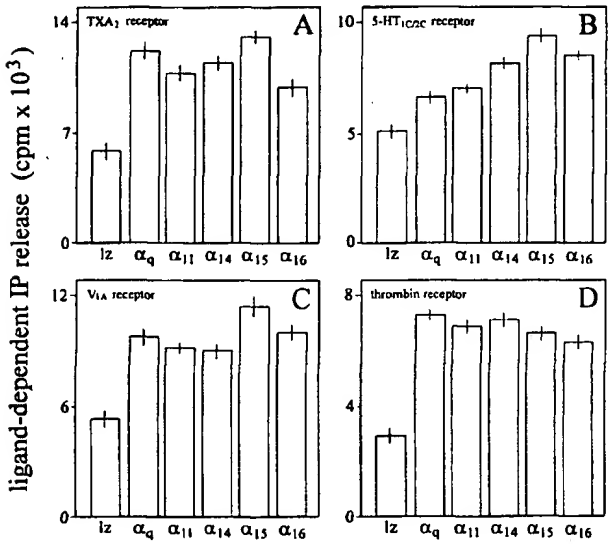

FIG. 6. **Formation of inositol phosphates in COS-7 cells coexpressing thromboxane A_2, 5-$HT_{1C/2C}$, vasopressin V_{1A} or thrombin receptors, and G-protein α-subunits.** COS-7 cells were cotransfected with cDNAs encoding the thromboxane A_2 receptor (*A*), the 5-$HT_{1C/2C}$ (*B*), the vasopressin V_{1A} receptor (*C*), or the thrombin receptor (*D*) and cDNAs encoding β-galactosidase (*lz*) or α-subunits of the $G\alpha_q$ family, $G\alpha_q$ (α_q), $G\alpha_{11}$ (α_{11}), $G\alpha_{14}$ (α_{14}), $G\alpha_{15}$ (α_{15}), and $G\alpha_{16}$ (α_{16}) as described under "Experimental Procedures." Cells were then incubated in the absence or presence of 3 μM U46619 (*A*), 10 μM serotonin (*B*), 1 μM [Arg^8]vasopressin (*C*), or 3 units/ml thrombin (*D*), and the ligand-dependent inositol phosphate formation was determined as described. Shown are mean values of triplicates ± S.D.

interact with the C5a receptor, which couples to $G\alpha_{16}$ but not to $G\alpha_{11}$ (51). This study suggests that the carboxyl-terminal 133 amino acids of $G\alpha_{16}$ do not alone account for its ability to interact with the C5a receptor, and that multiple regions of $G\alpha_{16}$ are responsible for the functional difference between $G\alpha_{11}$ and $G\alpha_{16}$, including a segment which comprises residues 220–240 of $G\alpha_{16}$. Thus, there are obviously many domains of $G\alpha_{15}$ and $G\alpha_{16}$ which directly or indirectly may affect the structure of G-protein and in this way may modulate the specificity of receptor G-protein interaction.

The fact that $G\alpha_{15}$ and $G\alpha_{16}$ interact with a wide variety of receptors can be useful for understanding the molecular details of the receptor G-protein interaction, once more structural data pertaining to receptors and different G-protein α-subunits are available. In addition, $G\alpha_{15/16}$ promiscuity may facilitate the examination of orphan receptors whose ligands and signal transduction properties are unknown. Cotransfection of orphan receptors and $G\alpha_{15}$ or $G\alpha_{16}$ into COS cells and subsequent determination of phospholipase C activity can be a way to search for ligands independent of the physiological signaling properties of the receptor.

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($n = 5$), respectively (not significant; $P = 0.19$, paired t-test). This strongly suggests that the synaptic potentiation in Fig. 4*A*, *C* was a result of Ca^{2+} entry through Ca^{2+}-permeable AMPA receptors.

One of the most prominent distinctions between Ca^{2+}-permeable AMPA receptors and the more well known Ca^{2+}-permeable glutamate receptors, the NMDA receptors, is that the latter are subject to a voltage-dependent block by Mg^{2+}, whereas Ca^{2+}-permeable AMPA receptors are not. Consequently, the synaptic Ca^{2+} fluxes associated with activation of Ca^{2+}-permeable AMPA receptors will be prominent at negative membrane potentials where the driving force on Ca^{2+} is high[24], whereas depolarized membrane potentials will favour strong Ca^{2+} fluxes through NMDA receptors[24,25]. Thus synaptic Ca^{2+}-permeable AMPA receptors may be expected to affect synaptic strength with a different sensitivity to activity than NMDA receptors. The synaptic strengthening that occurs following Ca^{2+} entry through Ca^{2+}-permeable AMPA receptors suggests that these channels are likely to provide an important physiological signal for triggering changes in synaptic transmission. □

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Transduction of bitter and sweet taste by gustducin

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SEVERAL lines of evidence suggest that both sweet and bitter tastes are transduced via receptors coupled to heterotrimeric guanine-nucleotide-binding proteins (G proteins) (reviewed in refs 1, 2). Gustducin is a taste receptor cell (TRC)-specific G protein that is closely related to the transducins[3]. Gustducin and rod transducin, which is also expressed in TRCs (ref. 4), have been proposed to couple bitter-responsive receptors to TRC-specific phosphodiesterases to regulate intracellular cyclic nucleotides[2–5]. Here we investigate gustducin's role in taste transduction by generating and characterizing mice deficient in the gustducin α-subunit (α-gustducin). As predicted, the mutant mice showed reduced behavioural and electrophysiological responses to bitter compounds, whereas they were indistinguishable from wild-type controls in their responses to salty and sour stimuli. Unexpectedly, mutant mice also exhibited reduced behavioural and electrophysiological responses to sweet compounds. Our results suggest that gustducin is a principal mediator of both bitter and sweet signal transduction.

Gene replacement was used to generate a null mutation of the α-gustducin gene. The murine α-gustducin gene was cloned and sequences surrounding the first protein coding exon were used to create the targeting vector (Fig. 1*a*). Positive/negative selection[6] was used to enrich for embryonic stem (ES) cell clones with a homologously recombined α-gustducin allele (Fig. 1). Chimaeric mice, generated from these ES cells by blastocyst injection, were back-crossed to C57BL/6J mice. Homozygous mice harbouring the recombined gustducin allele, genetically (C57BL/6J × 129/SvEmsJ)F_2, were produced by intercrossing heterozygous animals (Fig. 1*b*). Heterozygous and homozygous null mice were viable, healthy and fertile.

Taste epithelia from homozygous null mice were morphologically indistinguishable from epithelia of wild-type littermates (Fig. 1*c–f*). Mice have three types of taste papillae: fungiform, scattered throughout the anterior two thirds of the tongue; foliate, in lateral grooves; and a single circumvallate, at the back of the tongue[7]. The null mice had all three types of taste papillae, with an appropriate number of taste buds in each papilla and a normal complement of TRCs per bud.

α-Gustducin messenger RNA is normally expressed in TRCs of circumvallate, foliate and fungiform taste papillae[3]. However, in the null mice, α-gustducin expression was not detectable in the TRCs of circumvallate (compare Fig. 1*c* and *e*), foliate or fungiform taste papillae (data not shown). The sense probe controls (Fig. 1*d,f*) showed no hybridization to lingual tissue. We conclude that the targeting event resulted in a null allele of α-gustducin.

Forty-eight-hour two-bottle preference tests[8] were used to compare the taste responses of α-gustducin null mice with those of their wild-type siblings. A preference ratio (tastant solution consumed as a fraction of total liquid consumed) was calculated for each animal at each concentration. Tastants that are primarily salty (NaCl), sour (HCl), bitter (denatonium benzoate and quinine sulphate) or sweet (sucrose and the highly potent guanidine sweetener SC45647) to humans were tested. Two-factor (strain × concentration) analyses of variance (ANOVAs) (Table 1) were used to determine whether wild-type and null mice differed in their behavioural responses to tastants.

Responses of null mice to a concentration series of NaCl were similar to those of wild-type mice. Likewise, no strain difference was evident in the responses to a range of HCl solutions (Table 1; Fig. 2*a, b*). In both cases, all mice were indifferent to initial (low) tastant concentrations and exhibited aversive responses to higher concentrations. These data demonstrate that salty and sour behavioural responses are unaffected by the absence of α-gustducin.

In contrast, aversive responses to two bitter substances, denatonium benzoate and quinine sulphate, were diminished in the null mice compared to wild-type siblings (Table 1: Fig. 2*c, d*). Wild-type mice began to avoid denatonium at the 100 μM concentration, whereas null mice remained indifferent to denatonium even at 1 mM. The null mice required a ~40-fold higher concentration of denatonium and a ~100-fold higher concentration of

quinine than did their wild-type siblings to elicit comparable aversive responses. These data demonstrate that avoidance responses to these two bitter compounds are profoundly diminished in the absence of α-gustducin.

Unexpectedly, preference responses to two sweet substances, sucrose (Fig. 2*e*) and the sweetener SC45647 (ref. 9) (Fig. 2*f*) were reduced in the null mice compared to wild-type siblings (Table 1). Wild-type mice preferred sucrose at $\geqslant 50$ mM concentrations. However, the null mice remained indifferent at sucrose concentrations below 500 mM and required a ~17-fold higher concentration of sucrose than did their wild-type siblings to elicit comparable preference responses. Similar strain differences were found with SC45647 (Fig. 2*f*). Wild-type mice preferred SC45647 at 0.1 mM and 0.5 mM, whereas null mice remained indifferent at both of these concentrations. Additional testing indicated that the null mice preferred 2 mM SC45647 (preference ratio = 0.71; data not shown). We conclude that preference responses to these two sweet compounds are profoundly diminished in the absence of α-gustducin.

To confirm that the behavioural deficit in the mutants was due to a peripheral taste defect, we performed recordings from the chorda tympani branch of the facial (VII) nerve in null and wild-type mice. The chorda tympani innervates the anterior tongue and is responsive to salty, sweet, sour and bitter stimuli[10]. Summated chorda tympani responses were recorded during lingual stimulation with NaCl, HCl, quinine sulphate, denatonium benzoate, sucrose and SC45647 (sample traces shown, Fig. 3*a*, *d*, *g*). Each response was quantified by measuring two parameters: (1) the maximum amplitude of the neural response within four seconds of stimulus onset (phasic response), and (2) the response magnitude occurring ten seconds after stimulus onset (tonic response). The phasic response represents the initial nerve response to tastant stimulation of TRCs. The tonic response represents the sustained nerve response to continuous tastant stimulation of TRCs. Chorda tympani responses were normalized to the mean phasic response to NH_4Cl: only normalized phasic responses are shown (Fig. 3*b*,*c*,*e*,*f*,*h*,*i*). Two-factor (strain × concentration) ANOVAs were used to detect differences in the chorda tympani responses of null and wild-type mice (Table 1).

Both phasic and tonic responses of null mice were similar to

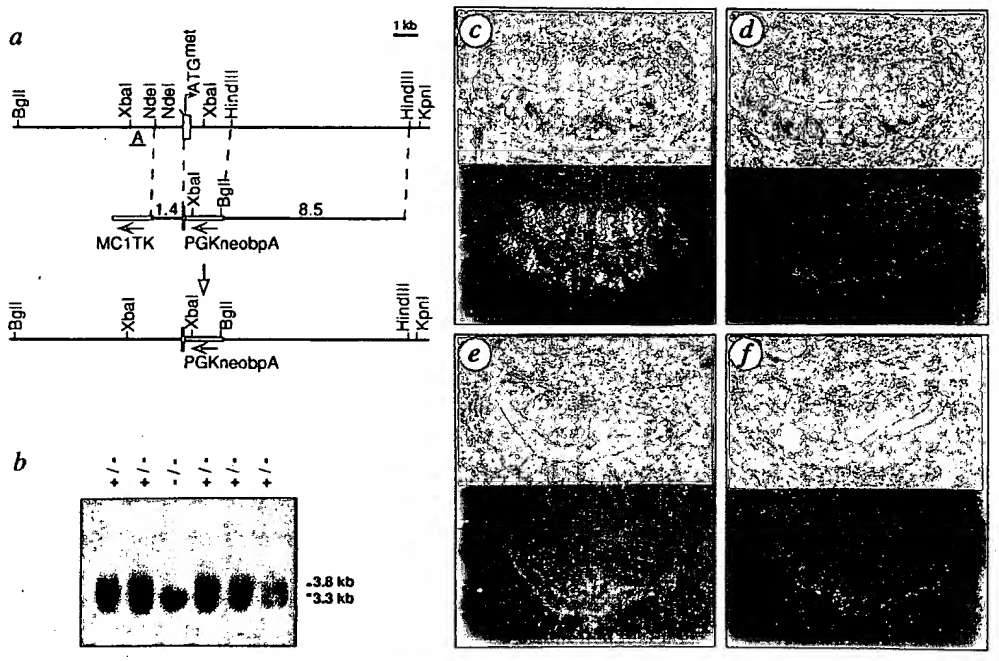

FIG. 1 Generation of α-gustducin null mice. *a*, Top, Map of the murine α-gustducin gene showing the first exon (open/filled box): 5′ untranslated leader sequence (open box), coding region for first 18 amino acids (filled box), and initiation codon (ATG^{met}). The probe used to screen G418-resistant ES cell clones is indicated (line A below map). Middle, map of the targeting vector, indicating the MC1*tk* and PGK*neo*bpA genes. The direction of transcription is indicated. The gustducin-homologous 5′ region is a 1.4-kb *Nde*I fragment which includes the transcription initiation sequences and 30 nucleotides (nt) of untranslated leader sequence from exon 1 (open box). The 3′ homologous region is an 8.5-kb *Hin*dIII fragment derived from the first intron. Bottom, map of the predicted structure of the homologously recombined gustducin allele, showing the presence of the first exon (open box) immediately preceding the PGK*neo* gene. *b*, Genomic analysis of offspring from a (+/−) × (+/−) mating. *Xba*I-digested genomic DNAs purified form tail biopsies were screened with probe A (see *a*). The wild-type α-gustducin restriction fragment is 3.8 kb. The targeting event generates a novel 3.3-kb fragment which includes a portion of the first exon fused to the *neo* gene. The third lane (−/−) identifies a homozygous null mouse. *c*–*f*, Photomicrographs of frozen sections of mouse taste papillae hybridized with ^{33}P-labelled murine gustducin probe and counterstained with haematoxylin–eosin. Bright-field (upper panels) and dark-field (lower panels) images are shown of antisense probe hybridized to circumvallate papillae from wild-type (*c*) and α-gustducin null mice (*e*), and of control sense probe hybridized to circumvallate papillae from wild-type (*d*) and α-gustducin null mice (*f*).

METHODS. The murine α-gustducin gene was cloned from a 129/Sv mouse genomic lambda library (Lamda FIX II, Stratagene) using rat α-gustducin cDNA[3] as probe. One clone, λ4, which contained the first protein-coding exon, was mapped and partially sequenced. A 1.4-kb *Nde*I fragment containing sequences homologous with the 5′-most sequence of the rat α-gustducin cDNA and an 8.5-kb *Hin*dIII fragment derived from the first intron were subcloned into a *neo*/*tk* selection vector. The resulting targeting vector DNA was linearized with *Sal*I before electroporation. Positive/negative selection[6] was used to obtain an allele of α-gustducin in which the first protein coding region (including the ATG initiation codon, and the splice donor sequences) was replaced with the PGK-*neo* gene[22] oriented opposite to gustducin's direction of transcription. The targeting vector also included the herpes simplex virus thymidine kinase (*tk*) gene for negative selection[23]. Transformation of the ES cell line W9.5 (ref. 24) was done as described[25] using *Sal*I-linearized α-gustducin targeting vector. Electroporation, selection and expansion of G418- and FIAU-resistant ES cell colonies were done as described[26]. ES cell and tail biopsy DNAs were prepared as described[27]. DNA (~5 μg) was digested with *Xba*I, electrophoresed and electroblotted as described[25]. Membranes were hybridized as described[25] with a radiolabelled 0.7-kb *Bgl*II–*Xba*I α-gustducin probe (probe A), consisting of unique sequences (data not shown) upstream from the 5′-most sequences present in the targeting vector. Southern analysis of genomic DNA from clonally expanded ES cell colonies indicated a homologous recombination frequency of 7%. ES cell clones containing a targeted gustducin allele were then karyotyped. Two independent clones with 40XY karyotypes were used to produce chimaeric mice by blastocyst injection[25]. Chimaeric males were bred to C57BL/6J females. Heterozygous progeny containing the mutant α-gustducin allele were intercrossed. Both lines yielded homozygous α-gustducin null pups with ~25% frequency. We used male mice (both −/− and +/+) with ~50/50 mix of 129/SvEMsJ and C57BL/6J backgrounds. For expression analysis, T3 and T7 RNA polymerases were used to generate ^{33}P-labelled RNA probes[28] from mouse gustducin exon-8 sequences subcloned into pBluescriptII. Transcription reactions containing 250 μCi ^{33}P-labelled thio-UTP were done according to the manufacturer's instructions. (Promega). Frozen sections (10 μm) of wild-type or α-gustducin null mouse tongues were hybridized with RNA probes (1×10^6 c.p.m. per section). Slides were processed as described[3,29].

those of wild-type mice for a concentration series of NaCl (Table 1; Fig. 3*b*). Similarly, no differences were seen with HCl solutions (Table 1; Fig. 3*c*). The electrophysiological results with NaCl and HCl are consistent with the behavioural results (Fig. 2) and indicate that α-gustducin null mice are unaltered in their ability to detect salty and sour stimuli. In contrast, but again consistent with the behavioural data, they showed reduced electrophysiological responses to bitter substances; compared to normal controls, mutants showed reduced responses to both denatonium benzoate and quinine, apparent in both phasic and tonic responses (Table 1; Fig. 3*e*, *f*). Similarly, the electrophysiological responses to the sweet compounds sucrose and SC45647 were significantly lower than those of wild-type mice (Table 1; Fig. 3*h*, *i*). Indeed, the mutant mice showed almost no electrophysiological response to either compound, even at concentrations that are intensely sweet to humans.

Taken together, our behavioural and electrophysiological results indicate that α-gustducin is a principal mediator of bitter and sweet taste transduction, although it is not involved in salty or sour transduction. At high concentrations, however, α-gustducin-deficient mice show a residual behavioural response to both bitter and sweet compounds, suggesting that additional G proteins may also be involved (candidate G proteins expressed in TRCs include G_s, G_{i-3}, G_{14} and rod transducin). Non-gustatory factors may also contribute to these behavioural responses. Interestingly, although the effect of the null mutation on the chorda tympani response was greater for sweet than for bitter compounds, the behavioural effect was greater with bitter compounds. Behavioural responses to bitter compounds may depend more on the glossopharyngeal nerve than on the chorda tympani, because the former contains a larger proportion of bitter-specific fibres[11]. Consistent with this idea, preliminary glossopharyngeal nerve recordings demonstrated profoundly diminished responses of the null mice to quinine sulphate and denatonium benzoate (K.S.G., Y. Ninomiya, G.T.W. and R.F.M., unpublished).

Several lines of evidence implicate G proteins and their coupled receptors in the transduction of bitter and sweet tastes (reviewed in refs 1,2). Denatonium increases inositol trisphosphate levels in mouse taste tissue[12] and leads to the release of intracellular Ca^{2+} from rat TRCs (ref. 13). Bitter transduction may involve activation of phospholipase C by gustducin's $\beta\gamma$ subunits and/or by an α_q-like subunit (α_{14} for example). Sucrose causes a GTP-dependent increase in taste tissue cAMP, apparently by activating adenylyl cyclase[14–16]. Microinjection of cyclic AMP into TRCs (ref. 17) or the addition of membrane permeant cyclic nucleotides[18,19] leads to inactivation of K^+ channels and TRC depolarization. Genetic

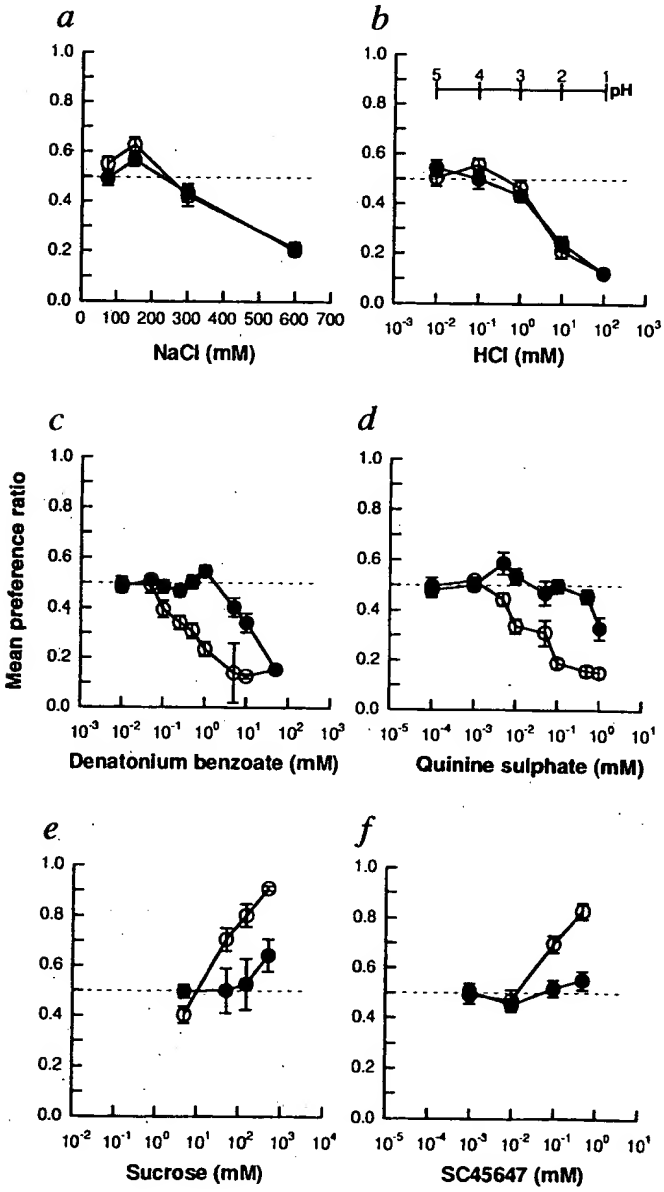

FIG. 2 Mean preference ratios from 48-h two-bottle (tastant versus distilled water) preference tests of male wild-type (open circles) and null (filled circles) mice. Tastant concentrations are listed in order of presentation. *a*, Behavioural responses to NaCl (75, 150, 300 and 600 mM) of wild-type mice ($n = 11$) and their null siblings ($n = 11$). No significant strain or interaction effects were found (P values were >0.05; Table 1). *b*, Behavioural responses to HCl (0.01, 0.1, 10 and 100 mM; pH range of 5.0 to 1.0) of wild-type mice ($n = 10–12$) and their null siblings ($n = 10–12$). No significant strain or interaction effects were found (P values were >0.05; Table 1). *c*, Behavioural responses to denatonium benzoate (0.01, 0.05, 0.1, 0.25, 0.5, 1.0, 5.0, 10.0, 50.0 mM) of wild-type mice ($n = 10–11$) and their null siblings ($n = 10–11$). Results of *t*-tests are as follows: 0.25 mM ($P < 0.01$); 0.5–10.0 mM ($P < 0.0001$). *d*, Behavioural responses to quinine sulphate (0.1, 1.0, 5.0, 10.0, 50.0, 100.0 μM; 0.5, 1.0 mM) of wild-type mice ($n = 9–12$) and their null siblings ($n = 10–12$). Results of *t*-tests are as follows: 5.0, 50.0 μM, 1.0 mM ($P < 0.05$); 10.0, 100, 500 μM ($P < 0.01$). *e*, Behavioural responses to sucrose (5, 50, 150 and 500 mM) of wild-type mice ($n = 7–12$) and their null siblings ($n = 11–12$). Results of *t*-tests are as follows: 50–500 mM ($P < 0.001$). *f*, Behavioural responses to SC45647 (1, 10, 100 and 500 μM) of wild-type mice ($n = 10–12$) and their null siblings ($n = 10–12$). Results of *t*-tests are as follows: 100 and 500 μM ($P < 0.001$).

METHODS. Polymerase chain reaction (PCR) was used to analyse male mice from heterozygous sib matings. Wild-type (+/+) and α-gustducin null (−/−) male siblings were used for behavioural tests. Both wild-type and null mice were genetically (C57BL/6J × 129/SvEmsJ)F_2. Three sets of wild-type and null siblings were tested. One set ($n = 11$ for each genotype) was tested with denatonium benzoate and NaCl. The second set ($n = 12$ for each genotype) was tested with quinine sulphate and sucrose. The third set ($n = 12$ for each genotype) was tested with sucrose, HCl, and SC45647. Between tests, mice were provided with acidified water (pH 4.2–5.2) for ~2-week intervals. Tested mice ranged in age from 8–20 weeks. Mice were individually housed, provided with food *ad libitum* (Pico Lab Mouse Diet 20 no. 5058; PMI Seeds) and presented with distilled water in two sipper bottles for 48 h before testing. During each 48-h test period, a given concentration of tastant was provided in one sipper bottle and the other contained distilled water. After 24 h, volumes consumed were recorded, the bottles refilled, and positions reversed (to control for positional cues that might affect preference). Tastants were presented in ascending series. Preference ratios were calculated as the fraction of tastant consumed compared to total volume consumed. *N* values varied owing to accidental drainage of the sipper tubes when mice inserted bedding into sipper spouts. When this occurred, data for that concentration point were omitted. ANOVAs (strain × concentration) (Table 1) were performed on the data, excluding those animals not generating data at all concentrations. Mean preference ratios shown in graphs and independent-group *t*-tests were calculated from total collected data.

ablation of α-gustducin would block the coupling of bitter and/or sweet responsive receptors to the regulation of certain intracellular second messengers, thereby blocking bitter/sweet TRC responses such as depolarization and neurotransmitter release. Any bitter or sweet compound that acts primarily on an α-gustducin-coupled taste receptor would be expected to have diminished behavioural and electrophysiological effects in the absence of α-gustducin. Compounds that act primarily via other G proteins or that by-pass taste receptors to act directly on intracellular targets[20] would not be expected to be affected by α-gustducin deficiency. The absence of the α subunit of gustducin may also have significant secondary effects on TRCs, for instance by creating an excess of free $\beta\gamma$ gustducin subunits that may inhibit other G-protein α-subunits or directly regulate effector enzymes[21]. Presumably, sweet-responsive versus bitter-responsive TRCs containing gustducin are physically distinct cells. Alternatively, the same gustducin-positive TRC may respond to both sweet and bitter, but via different transduction pathways. Ultimately, direct electrophysiological recording from the gustducin lineage of

TABLE 1 Summary of analyses of variance (strain × concentration) on behavioural and neurophysiological data

Response		Preference*		Phasic response†		Tonic response‡	
Tastant	Effect	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
NaCl	S:	0.687	0.4171	0.060	0.8103	0.066	0.8003
	C:	134.017	0.0000§	79.623	0.0000	202.547	0.0000
	SC:	1.651	0.1871	1.850	0.1760	1.642	0.2117
HCl	S:	0.416	0.5271	0.498	0.4930	0.560	0.4677
	C:	118.852	0.0000	132.368	0.0000	27.505	0.0000
	SC:	1.040	0.3929	1.495	0.2429	0.080	0.9238
Denatonium	S:	68.614	0.0000	34.827	0.0000	15.754	0.0014
	C:	45.691	0.0000	80.897	0.0000	6.478	0.0011
	SC:	10.384	0.0000	7.954	0.0003	3.136	0.0353
QSO_4	S:	32.204	0.0000	5.806	0.0304	7.045	0.0189
	C:	15.278	0.0000	114.061	0.0000	13.504	0.0001
	SC:	6.580	0.0000	5.486	0.0098	5.607	0.0090
Sucrose	S:	30.534	0.0001	14.569	0.0021	21.265	0.0005
	C:	21.520	0.0000	22.692	0.0000	20.775	0.0000
	SC:	10.293	0.0000	16.612	0.0000	17.653	0.0000
SC45647	S:	21.211	0.0002	42.066	0.0000	39.029	0.0000
	C:	20.294	0.0000	45.181	0.0000	20.422	0.0000
	SC:	8.707	0.0001	25.342	0.0000	23.294	0.0000

* S, strain; C, concentration; SC, strain × concentration. Based on data shown in Fig. 2.
† Based on data shown in Fig. 3.
‡ Based on data not shown.
§ *P* values <0.00005 are presented as *P* = 0.0000.

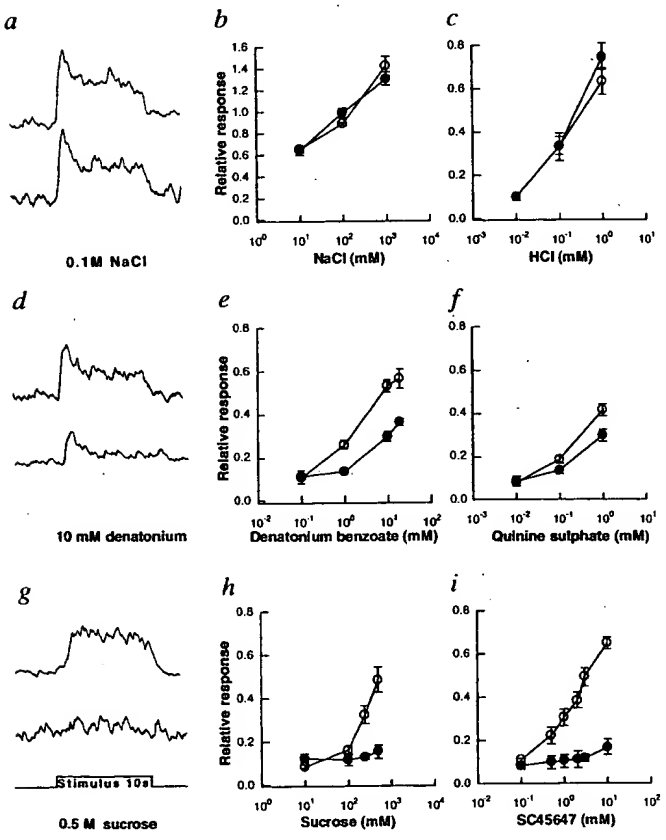

FIG. 3 Chorda tympani responses to lingual application of taste stimuli for wild-type control and α-gustducin null mice. *a*, *d*, *g*, Integrated neural responses of a wild-type (upper trace) and a null mouse (lower trace) to 0.1 M NaCl (*a*), 10 mM denatonium benzoate (*d*) and 0.5 M sucrose (*g*). *b*, *c*, *e*, *f*, *h*, *i*, phasic responses of null ($n = 6$, filled circles) and wild-type mice ($n = 9$–10, open circles) to taste compounds. Data shown represent the maximum neural response amplitude occurring within 4 seconds of stimulus onset and are expressed relative to the response to 0.1 M NH_4Cl. Owing to the large response of the chorda tympani to NaCl, the *y*-axis of *b* ranges from 0 to 1.4; *y*-axes for the other tastants range from 0 to 0.8. *b*, Neural response to NaCl concentrations (0.01, 0.1, 1.0 M). No significant strain or interaction effects were present (*P* values > 0.05; Table 1). *c*, Neural response to HCl concentrations (0.01, 0.1, 1.0 mM). No significant strain or interaction effects were present (*P* values >0.05; Table 1). *e*, Neural responses to denatonium benzoate concentrations (0.1, 1.0, 10.0, 20.0 mM). Results of *t*-tests are as follows: 1.0 mM ($P < 0.001$), 10.0 mM ($P < 0.0001$), 20 mM ($P < 0.01$). *f*, Neural response to quinine sulphate concentrations (0.01, 0.1, 1.0 mM). Results of *t*-tests are as follows: 0.1 mM ($P < 0.05$), 1.0 mM ($P < 0.01$). *h*, Neural response to sucrose concentrations (0.01, 0.1, 0.25, 0.5 M). Results of *t*-tests are as follows: 0.25 ($P < 0.01$), 0.5 M ($P < 0.0001$). *i*, Neural response to SC45647 concentrations (0.1, 0.5, 1.0, 2.0, 3.0, 10.0 mM). Results of *t*-tests are as follows: 0.5 mM ($P < 0.05$); 1.0 mM ($P < 0.01$), 2.0 mM ($P < 0.001$), 3.0–10 mM ($P < 0.0001$).

METHODS. Male mice (Fig. 2 legend) were anaesthetized with sodium pentobarbital (60 mg kg^{-1}). Surgical, taste stimulation and recording procedures have been described[30]. Neural signals were amplified (10,000×) and integrated using an RMS-DC converter (Hendrick and Associates, Tallahassee, FL) with a time constant of 0.5 s. Taste solutions were presented for 10 s followed by a ⩾1-min rinse with distilled water at a constant flow rate of 3 ml min^{-1}. Excess fluid was removed through a suction tube positioned beneath the tip of the tongue. Each tastant concentration was presented at least twice and the mean response calculated. Tastants were presented as an ascending concentration series with presentation of 0.1 M NH_4Cl bracketing each concentration series. The mean phasic response to 0.1 M NH_4Cl was used to normalize responses within each bracketed concentration series. This concentration of NH_4Cl produced similar chorda tympani response magnitudes in null and wild-type mice. Data were analysed using strain × concentration ANOVAs for each tastant (see text and Table 1). *Post hoc* comparisons were performed using independent group *t*-tests (Fig. 3 legend).

TRCs from wild-type and mutant mice with α-gustducin point mutations may enable us to determine gustducin's specific roles in bitter and sweet transduction. □

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Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate

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CERAMIDE is an important regulatory participant of programmed cell death (apoptosis) induced by tumour-necrosis factor (TNF)-α and Fas ligand, members of the TNF superfamily[1–6]. Conversely, sphingosine and sphingosine-1-phosphate, which are metabolites of ceramide, induce mitogenesis[7] and have been implicated as second messengers in cellular proliferation induced by platelet-derived growth factor and serum[8,9]. Here we report that sphingosine-1-phosphate prevents the appearance of the key features of apoptosis, namely intranucleosomal DNA fragmentation and morphological changes, which result from increased concentrations of ceramide. Furthermore, inhibition of ceramide-mediated apoptosis by activation of protein kinase C results from stimulation of sphingosine kinase and the concomitant increase in intracellular sphingosine-1-phosphate. Finally sphingosine-1-phosphate not only stimulates the extracellular signal-regulated kinase (ERK) pathway[10], it counteracts the ceramide-induced activation of stress-activated protein kinase (SAPK/JNK). Thus, the balance between the intracellular levels of ceramide and sphingosine-1-phosphate and their regulatory effects on different family members of mitogen-activated protein kinases determines the fate of the cell.

Consistent with previous studies[4,11], increasing intracellular levels of ceramide in human promyelocytic HL-60 cells or U937 monoblastic leukaemia cells either by addition of the membrane-permeable ceramide analogue C_2-ceramide (Fig. 1*a–d*), or by treatment with sphingomyelinase (Fig. 1*e–f*), induced apoptosis. Exposure to sphingosine-1-phosphate (SPP) prevented ceramide-induced apoptosis in both cell lines, as measured by oligonucleosomal DNA fragment electrophoresis (Fig. 1*a*, *e*) and a quantitative DNA fragmentation assay (Fig. 1*f*). SPP also markedly reduced genomic fragmentation and the morphological changes associated with apoptosis, as detected by *in situ* staining (Fig. 1*b*, *d*). The ability of SPP to prevent apoptosis induced by serum deprivation in HL-60 cells (Fig. 1*a*) may also be related to an attenuation of ceramide-mediated cell death, given that serum withdrawal results in marked accumulation of ceramide[12].

Because the binding of TNF-α and the Fas ligand to their receptors results in stimulation of sphingomyelinase, which catalyses the degradation of sphingomyelin to ceramide[1–5,13–15], we investigated the effects of SPP on apoptosis induced by these cytokines. TNF-α not only induced intranucleosomal DNA fragmentation in U937 cells (Fig. 2*a*), it also reduced survival, as determined by the 3-[4,5-dimethylthiazol-2-yl-]2,5-diphenyl tetrazolium bromide (MTT) dye-reduction assay (Fig. 2*b*), effects that were prevented by co-treatment with SPP. SPP also reduced TNF-induced DNA strand breaks and the expression of the apoptotic traits (Fig. 2*c–f*). In addition, SPP inhibited apoptosis induced by Fas ligation in Fas-expressing Jurkat T cells (Fig. 3*c*).

Activation of protein kinase C antagonizes apoptosis induced by TNF-α, Fas ligand, and ionizing radiation, suggesting that the diacylglycerol/protein kinase C pathway counteracts ceramide-mediated apoptosis[4,11,15,16]. Moreover, inhibitors of protein kinase C induce apoptosis in haematopoietic and neoplastic cell lines[17]. Although the mechanism by which protein kinase C opposes ceramide-mediated apoptosis has not been determined, activation of this enzyme in diverse cell types stimulates sphingosine kinase activity, resulting in intracellular accumulation of SPP[7]. SPP prevented apoptosis induced by several inhibitors of protein kinase C, including the bisindolylmaleimide GF 109203X, H7, calphostin C, UCN-01 (7-hydroxystaurosporine), and chelerythrin, in Swiss 3T3 and U937 cells (Fig. 3*a*, *b*). To verify that the activation of sphingosine kinase by protein kinase C was responsible for inhibiting ceramide-mediated apoptosis, we used a competitive inhibitor of sphingosine kinase, *N,N*-dimethylsphingosine, which is a more potent inhibitor of sphingosine kinase than of protein kinase C[18] (B.K. and S.S., unpublished results). At a concentration that markedly inhibited sphingosine kinase activity and formation induced apoptosis and prevented the ability of the protein kinase C activator, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), to suppress DNA fragmentation induced by Fas ligation in Jurkat T cells (Fig. 3*c*). SPP restored the cytoprotective activity of TPA and counteracted the DNA fragmentation induced by *N,N*-dimethylsphingosine (Fig. 3*c*). Results were similar with